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Major global changes interact to cause male-biased sex ratios in a reptile with temperature-dependent sex determination

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ABSTRACT

Habitat loss and pollution are two of the greatest global threats to biodiversity. Due to their widespread prevalence, these threats often co-occur, yet their interactive effects on organisms remain poorly understood. Some reptiles are vulnerable to these threats because they have specific microclimate requirements for embryonic development and because pollutants are maternally transferred to their eggs; both incubation temperature and pollutants affect reptile sex determination. In aquatic turtles, females often select nest sites in recently planted agricultural fields but the impact of nesting in polluted agricultural habitats is not understood. We examined the influences of crop agriculture and mercury pollution on nest microclimate and offspring sex ratios of *Chelydra serpentina*. We hypothesized that crop growth in agricultural fields would shade and cool turtle nests, decrease moisture levels, increase male offspring production, and interact with maternally-derived mercury to impact sex determination. As predicted, nests shaded by crops had lower average temperatures (−2.5 °C) and moisture levels (−107 kPa) than control nests. In field and laboratory experiments, agricultural thermal regimens increased the proportion of male offspring in clutches and this effect was intensified in the presence of mercury. Global temperatures are expected to rise within the 21st century and to have a feminizing effect on reptiles with temperature-dependent sex determination. That prediction should be refined to incorporate how the cooling effect of some local habitat conditions (e.g., agricultural fields), and interactions between anthropogenic land-use and common pollutants, will interact with climate change to influence sex ratios of reptiles with temperature-dependent sex determination.

1. Introduction

Over the last 500 years, anthropogenic changes have led to the loss of over 600 vertebrate species, signaling the onset of the sixth mass extinction event (Ceballos et al., 2015). Among diverse global changes, habitat loss and environmental pollution are two of the greatest concerns for biodiversity (Meiri and Chapple, 2016). Despite the fact that pollution and habitat conversion for crop agriculture often co-occur (Matson et al., 1997), very little is known about how they interact to affect biodiversity.

Reptiles are a biologically diverse and highly threatened vertebrate group that are commonly subjected to the adverse reproductive effects of rapid global changes (Bland and Böhm, 2016; Böhm et al., 2016). Most reptiles (~85%) are oviparious and have specific microhabitat, temperature, and moisture requirements for successful embryonic development (Deeming, 2004). Many reptiles exhibit temperature-dependent sex determination (hereafter referred to as TSD); the temperature at which eggs are incubated is the primary determinant of the sex of hatchlings produced (Bull, 1980; Janzen, 1992). Anthropogenic habitat modifications such as dense agricultural crops that decrease solar exposure of nests are predicted to produce male-biased sex ratios (Freedberg et al., 2011; Mui et al., 2015). Conversely, rising global temperatures due to climate change may increase female-biased offspring sex ratios in animals with TSD (Mitchell and Janzen, 2010; Böhm et al., 2016). To make matters more complex, reptiles nesting in human altered landscapes also maternally transfer high levels of endocrine disrupting pollutants to their eggs that interfere with early development, sex determination, and decrease hatch success (Crews et al., 1995; Bishop et al., 1996; Guillette et al., 2000; Schneider, 2001). However, empirical tests of the above thermal predictions, as well how

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they interact with pollutants, are currently lacking.

In this study, we examined the effects of crop agriculture on nest microclimate, as well as how agriculturally-induced changes in nest microclimate act individually and interactively with mercury pollution, to impact offspring sex of a freshwater turtle with TSD. In numerous species of aquatic turtles, females select nest sites that have a high degree of solar exposure such as sandy, mossy, or rocky patches adjacent to rivers and streams. However, they are sometimes more attracted to areas where humans have disturbed terrestrial substrates and vegetation (e.g., road shoulders, recently logged areas, or recently tilled agricultural fields), increasing solar exposure and facilitating easier nest excavation (Kolbe and Janzen, 2002; Beaudry et al., 2010; Freedberg et al., 2011; Riley and Litzgus, 2013; Mui et al., 2015). The costs and benefits of nesting in these disturbed habitats are poorly understood. We focused on co-exposure to mercury because it is one of the most globally widespread contaminants (Driscoll et al., 2013), persists in the environment for hundreds-to-thousands of years (Selin, 2009), and has a suite of adverse effects on the endocrine, reproductive, and nervous systems of vertebrates (Rice et al., 2014). Because hormones are known to influence offspring sex and even reverse TSD in reptiles (Warner et al., 2009; Matsumoto and Crews, 2012; Merchant-Larios and Diaz-Hernandez, 2012), endocrine disrupting pollutants and early developmental temperatures may interact. Previous studies demonstrated that female turtles bioaccumulate and maternally transfer mercury to their eggs (Hopkins et al., 2013b). However, the effects of mercury on offspring sex ratios and the interactive effects of mercury and agricultural land use have not been studied.

To investigate the individual and interactive effects of mercury and crop agriculture, we used a 2 × 2 factorial design (presence or absence of agriculture and/or mercury) in coupled field and laboratory experiments. Our objectives were to:

(a) Describe the influence of agricultural land use on turtle nest microclimate (thermal and hydric characteristics),
(b) Determine the individual and interactive effects of agricultural land use and a wide-spread and persistent endocrine disrupting pollutant on offspring sex determination of turtles, *and*
(c) Integrate realistic laboratory simulations with manipulative field experiments to disentangle temperature from other potential mechanisms underlying observed effects on sex determination.

We hypothesized that in the weeks following nesting, rapid growth of crops over turtle nests in agricultural fields would shade and cool nests, reduce water content of nest substrates through evapotranspiration by plants, and increase the proportion of male offspring produced. We also postulated that high concentrations of maternally transferred mercury in eggs would interact with agricultural incubation conditions to exacerbate effects on offspring sex determination.

Our study provides valuable information for conservation efforts in an imperiled vertebrate group using a globally widespread habitat disturbance (crop fields) and pervasive environmental pollutant (mercury). It advances our understanding of thermal effects of anthropogenic land use on nest microclimate and sex ratios in reptiles with TSD by generating high resolution (hourly fluctuating) thermal profiles from the field to experimentally evaluate the role of realistic temperature fluctuations on offspring sex determination (Bowden et al., 2014). Our study is also the first to examine mercury impacts on offspring sex determination in a species with TSD, as well as the interactive effects of maternally-transferred mercury and incubation temperatures in agricultural fields on offspring sex (Freedberg et al., 2011; Bowden et al., 2014; Mui et al., 2015). Because reptiles with TSD are predicted to be vulnerable to feminizing effects of climate change, research that identifies how habitat composition (i.e., levels of vegetation and chemical pollution at nest sites) can alter sex ratios is needed to refine climate-related predictions.

2. Methods

2.1. Study system

We used the common snapping turtle (*Chelydra serpentina*) as our focal species. Due to their long life span, high trophic-level, and sedentary nature (Hammer, 1969; Punzo, 1975; Obbard and Brooks, 1981), tissue concentrations of mercury in snapping turtles can reach high levels (Hopkins et al., 2013a). From mid-May to the end of June, female snapping turtles leave their aquatic habitats to nest, often in agricultural fields (Freedberg et al., 2011; Mui et al., 2015; Thompson et al., 2017). Clutch sizes typically range from 26 to 55 eggs (Steyermark et al., 2008). In snapping turtles, females are produced at high and low temperatures and males at intermediate temperatures (Bull, 1980; Janzen, 1992). At constant temperatures in the laboratory, the upper pivotal temperature is 27.8 °C and the lower pivotal temperature is 21.4 °C. Under more variable temperatures in the field, the upper pivotal sex determining temperature may be closer to 25.5 °C (Bull, 1985).

Our study area was along the South River, Virginia, USA, an area subjected to both mercury pollution and intensive agricultural land use. A manufacturing plant leaked mercuric sulfate into this river from 1929 to 1959 (Carter, 1977). Considerable amounts of mercury remain in the river and the floodplain, and as a result, tissue concentrations of mercury in wildlife living downstream are high (Bouland et al., 2012; Todd et al., 2012; Drewett et al., 2013; Hopkins et al., 2013a). Land use within 200 m of the South River upstream and downstream of the mercury source is 61% and 68% agricultural, respectively (2011 National Land Cover Database; Multi-Resolution Land Characteristics Consortium, NLCD 2011).

We collected 43 entire clutches of eggs from nests located upstream (n = 20) and downstream (n = 23) from the initial mercury point source on the South River (Virginia DGIF Permit No. 048080, VT IACUC No. 14–110) from May–June 2014. We found nests mostly in agricultural fields (n = 38/43). Other nests were found along stream banks (n = 2/43), in commercial nursery properties (n = 2/43), and in a lawn (n = 1/43). Clutches were collected immediately following oviposition. We marked each egg with a unique identification number, weighed it to the nearest 0.01 g, and measured it to the nearest 0.01 mm.

2.2. Nest microclimate experimental design

We used experimental treatment plots to investigate the effects of crop growth on turtle nest microclimate. We constructed treatment plots in a field that was planted with corn in early May 2014 and that had been used by snapping turtles for nesting the previous year (> 20nests in < 6.0 Hectares; Thompson et al., 2017). We used a random sample (n = 10) of the 2013 nest sites as the locations of our experimental plots. All plots (n = 5 per treatment) were circles six meters in diameter and assigned to either a control treatment, referred to as “Open” (crops removed and non-crop vegetation limited to 10% cover), or an agricultural treatment referred to as “Shade” (crops were allowed to grow). Approximately 10% canopy cover over nests is typical of snapping turtle nest sites in non-agricultural areas (Janzen, 1994; Kolbe and Janzen, 2002).

We constructed one artificial nest at the center of each plot. The top and bottom of artificial nest chambers were 105 mm and 150 mm below the soil surface, respectively (Congdon et al., 1987). We standardized the clutch size in the nests to 34 artificial eggs to represent the average clutch size and mass at our study site (Hopkins et al., 2013b). Eggs were hollow polypropylene plastic balls with a diameter of 25 mm (CIC Ball Company, PN) filled with wire pulling lubricant (ClearGlide™) which closely mimics the thermal properties of amniotic eggs (Ardia et al., 2010; Coe et al., 2015). We placed Hobo-Temp temperature probes attached to cables (Onset Computer Corporation, Pocasset, Massachusetts) at the top and bottom of artificial nests and recorded temperature

at one hour intervals. We uploaded temperature data regularly throughout the duration of the study without disturbing the artificial nests (the Hobo-Temp logger sits above the soil surface). Turtles release bladder water while constructing their nests; we therefore sprayed the dirt with water (equivalent to ~4 ml) as we sealed nest chambers (Marchand and Litvaitis, 2004). We placed wire mesh predator guards over each artificial nest (Riley and Litzgus, 2013).

We measured vegetation growth in our experimental plots every eight days to relate changes in vegetative height, density, and cover to changes in hydric and thermal dynamics of nests. We established two perpendicular transects through each treatment plot and used the line-point intercept method (LPI) to quantify vegetative cover and height (Noon, 1981; Pilliod and Arkle, 2013). We recorded vegetation height every 40 cm (± 5 cm) along the two 6 m long transects. We calculated vegetative cover as:

$$\%\text{Cover} = \frac{\left(V1/16\right) + \left(V2/16\right)^{*}}{2} 100$$

where *V1* and *V2* are the number of points bisecting vegetation along transect 1 and 2, respectively. We estimated foliar density across each transect using a cover pole, and categorized density at each 10 cm height interval (Noon, 1981; Griffith and Youtie, 1998). Density poles were placed at a constant distance from a viewer who visually estimated the percent of each section of the pole obscured by vegetation using density scores. In this study, density scores for each 10 cm interval were categorized as follows: 0 = none, 1 = 0–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, 5 = 81–100% of visibility obscured, from a constant distance of 6 m and height of 170 cm. Zeros were not included in average height or density estimates; we use the term cover to describe the percent of plots covered in vegetation, but use height and density to describe how tall and thick the vegetation was only where it occurred.

We also determined soil water content and water potential in our treatment plots every eight days. We collected two soil samples from > 10 cm away from nests in random locations in plots using a hand held core sampler to the depth that corresponded to the center of our nest chambers (~13 cm). We determined water content by weighing soil samples and then drying them to a constant mass in an Isotemp drying oven (Fisher Scientific, Ottawa, ON, Canada) at 100 °C (Gardner, 1986); water content corresponds to the difference between these values. We converted water content to water potential using soil-water retention curves that we established using a Wescor Hygrometer/Psychrometer with a C-52 Sample Chamber for the two soil types present in our experimental plots (Soil Survey Geographic Database, Fig. 1). We used data from the National Oceanic and Atmospheric Association (NOAA) to identify the timing and the relative magnitude of rain events.

2.3. Sex ratio experimental design

We used a 2 × 2 factorial design (presence or absence of agricultural shade and/or mercury) to conduct simultaneous field and laboratory incubation experiments. We randomly assigned whole clutches to either the field incubation experiment (n = 18 clutches total; mercury contaminated = 10, reference = 8) or the laboratory incubation experiment (n = 25 clutches total; mercury contaminated = 13, reference = 12) as they were collected.

The first factor of our 2 × 2 design was agriculture treatment. We randomly split clutches collected from mercury contaminated and reference locations and assigned each half to an agriculture incubation treatment (Open or Shade). The second factor of our 2 × 2 design was a mercury treatment which had two treatment groups: eggs that contained high concentrations of maternally derived mercury, referred to as “Hg” (eggs collected from within 16 km *downstream* of the original mercury source), and eggs that only contained background

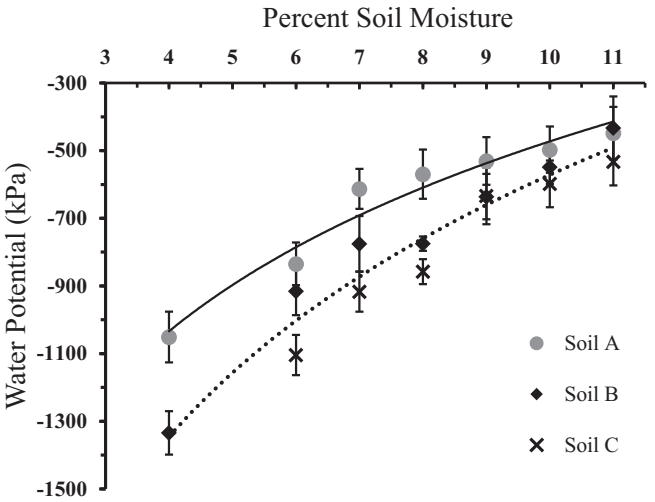

Fig. 1. Soil-water retention curves for the two distinct soil types at the study site. SSURGO characterizes the field as containing three types of fine sandy loams; Chavies (Soil A), Unisom (Soil B), and Buchanan (Soil C). However, we detected no difference in water holding capacity (water potential) among soils B and C, and consequently pooled data to construct one soil-water retention curve for both types (Retention II). A separate retention curve was fit for Soil A (Retention I). Retention I relates soil water moisture to water potential using the equation; y = 612.59ln(x) - 1883.1, with R^2 = 0.957. Retention II relates soil water moisture to water potential using the equation; y = 844.24ln(x) - 2515.4, with R^2 = 0.939.

concentrations of mercury, referred to as “Ref” (eggs collected 6–14 km *upstream* from the mercury source). Mark-recapture studies conducted at these sites for > 10 yrs. documented that turtles with markings from the Ref sites are not collected in the Hg sites (or vice versa; e.g., Hopkins et al., 2013a). We quantified mercury for each clutch from maternal blood or from one randomly selected egg from each clutch. Turtle eggs from the same clutch have similar maternally-derived trace element concentrations (Van Dyke et al., 2013). We used a regression equation to estimate egg mercury concentration from maternal blood concentration as these two variables are strongly and positively related (Hopkins et al., 2013a). We froze tissues at $-20\,^{\circ}C$ until mercury analysis. We lyophilized and homogenized the eggs and report their total mercury (THg) concentrations on a dry mass (dwt, in ppm) basis. The percent moisture of eggs was 75.5 ± 0.7% SE (n = 44). Samples were analyzed by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monoroe, CT, USA) following U.S. Environmental Protection Agency (U.S. EPA) method 7473 (USEPA 1998). Standard reference materials were analyzed alongside experimental samples; mean percent recoveries of THg for the standard reference materials were 104.3 ± 1.0% SE (n = 14) and 100.3 ± 0.3% SE (n = 14). All samples were run in duplicate; the average relative percent difference was 6.3% ± 1.2 SE.

Clutches assigned to the field experiment were transplanted into nests in the experimental plots and left undisturbed until retrieval just prior to their hatching. We constructed 3–4 nests in each plot, in cardinal directions 130 cm from the artificial nests in the center of each plot. We used the same methods to construct nests in the field experiment as we used for our artificial nests. However, we used iButton temperature data loggers instead of Hobo loggers to record thermal profiles in nests (DS1923, Embedded Data Systems, KY, USA). Prior to burying eggs, we standardized the clutch size to 34 by adding artificial eggs in order to standardize bias in thermal gradients within nests and to control for the impact of clutch size on the thermal inertia of nests. We placed wire mesh predator guards over each nest (Riley and Litzgus, 2013).

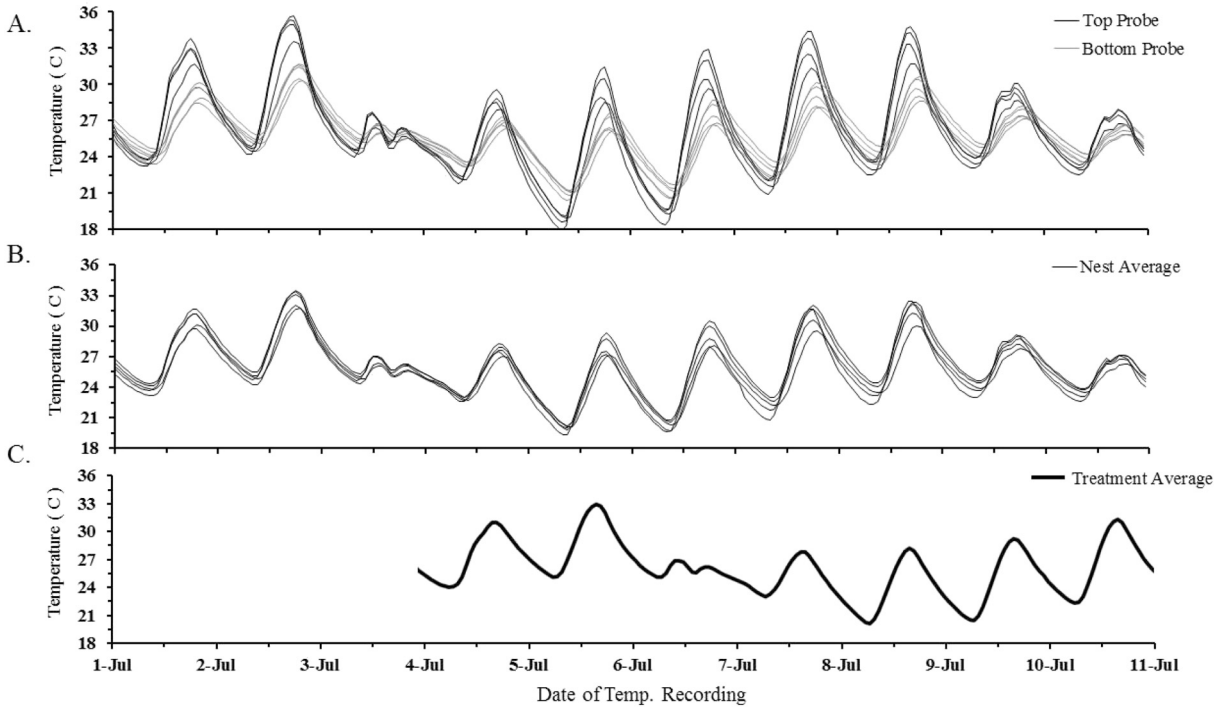

Fig. 2. Sampling design used to convert temperatures recorded in the field to a laboratory incubation regime using real data. Graph (A) shows 8 days of temperatures recorded at the top (black) and bottom (grey) of the artificial nests in the Open treatment ($N = 5$). Four days of temperature data were averaged for the top and bottom of each nest, for each hour (B). An overall average was taken from the five treatment replicates (C) and used for incubation in the lab experiment for the next four days.

We retrieved eggs from field nests as they neared completion of incubation, between August 18 and September 27. We then incubated the eggs under constant temperatures corresponding to the average nest temperature recorded in the field during the previous three weeks until they hatched (Open = 23.3 $^{\circ}C$, Shade = 20.5 $^{\circ}C$). During egg retrieval, we categorized the eggs by the order they were removed as we dug down into each nest chamber. We classified the first half of eggs removed as the top layer and the second half as the bottom layer. During retrieval, four of the 18 nests in the Open treatment plots had pipped; no eggs in any of the nests in Shade treatment plots had pipped. We used the retrieval date as the last day of incubation for the Open nests that pipped in the field. Based on embryonic staging at oviposition coupled with retrieval and hatch dates, all embryos spent > 90% of their total incubation in the field. Three of the 72 iButtons malfunctioned, resulting in a total 69 iButtons used to investigate the effect of our treatments on nest temperature and offspring sex determination (Top; $N = 34$, Bottom; $N = 35$).

We calculated incubation regimens for the laboratory experiment from temperatures recorded in the ten artificial nests (Fig. 2). Because the laboratory incubation experiment was running simultaneously as we generated our thermal profiles from artificial nests, we staggered the laboratory experiment four days behind the date when temperatures were recorded in the field (Thompson, 2017). Throughout the duration of incubation, we uploaded temperature data from the field every four days and used it to program the next four days of incubation in the lab. We split clutches assigned to the laboratory experiment between Open and Shade incubation treatments in Memmert IPP 55 Plus incubators ($n = 4$; 2 per treatment, Memmert GmbH + Co·KG, Schwabach, Germany). We calibrated the incubators prior to the study and monitored their accuracy throughout incubation using two iButton data loggers placed in the center of each incubator. Eggs were incubated ~5 cm away from one another and were ~80% covered by vermiculite in plastic containers (1:1, water: vermiculite) capped with perforated lids. We rehydrated and rotated egg containers among shelves every four days (Packard et al., 1987).

In both experiments, pipped eggs and hatchlings were placed in individual Tupperware™ containers with 2 cm of distilled water and kept at 23 $^{\circ}C$ in an environmental chamber. Six weeks post-hatch, we euthanized and determined the sex of each hatchling by examination of the gonads under a dissecting microscope (Yntema, 1968). At six weeks of age, gonadal morphology is well developed and sexes are markedly different.

2.4. Statistical methods

Treatment effects on offspring sex were analyzed using R software and the Ime4 package (R Foundation for Statistical Computing, Vienna, Austria). All other analyses were performed in JMP Pro 11 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel 2013. In all cases significance was assessed at $\alpha = 0.05$.

We quantified the impact of crops on the thermal and hydric dynamics of field nests throughout incubation using repeated measures ANOVA (RM ANOVA) that included the agriculture treatment as a between subjects effect and sample date as a within subject effect. For analyses involving water content and water potential, our RM ANOVAs used the average of the two soil core samples from plots as the experimental unit. The experimental unit for analyses of thermal responses (average daily temperature and average daily variance in °C) was each treatment plot ($n = 5$ plots/trt), calculated as the average of the field nests ($n = 3$–4) within each plot.

We also modeled the effect of our agriculture treatment on the thermal dynamics of field nests during the thermosensitive period (when sex is determined) using linear models (LMs). We calculated the thermosensitive period as the middle third of the incubation period (Yntema, 1979; Yntema and Mrosovsky, 1982). Our LMs included agriculture treatment, mercury treatment, and nest layer as fixed, categorical effects. Three way interactions were tested but were not significant ($p > 0.20$ in all cases) and were removed from our final models. The thermal responses we modeled were average daily temperature, thermal variability (using variance), and constant

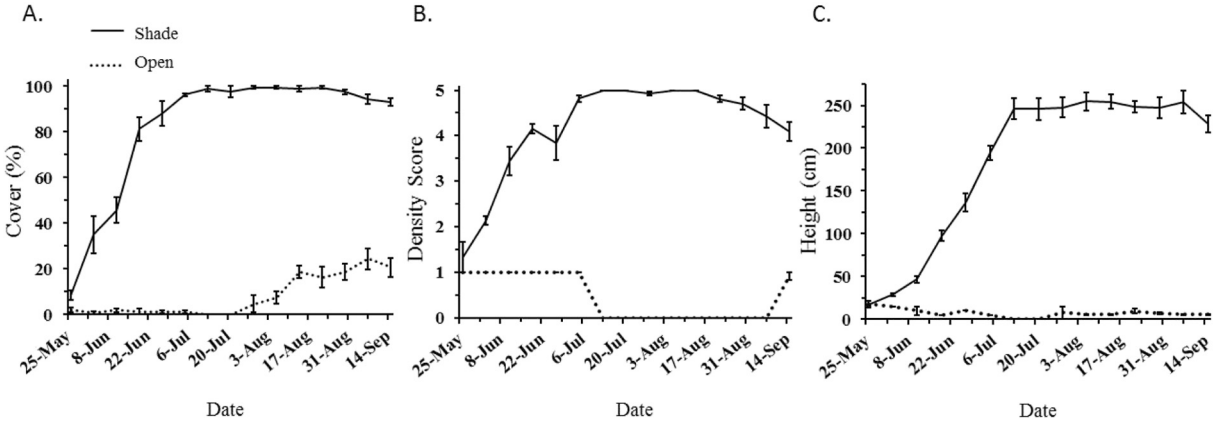

Fig. 3. Average vegetative cover (A), density (B), and height (C) for Open (dotted) and Shade (solid) treatment plots throughout the incubation period. Both crop and non-crop vegetation are included in average values.

temperature equivalent (hereafter referred to as CTE). Half of development occurs above and below the CTE (Georges et al., 2004); CTE account for faster development at higher temperatures and slower development at lower temperatures. We parameterized our CTE models using $T_0 = 16.01$ (Georges et al., 2004; Freedberg et al., 2011).

We investigated treatment effects on offspring sex using the dichotomous response of male v. female as the dependent variable. Models for both experiments included the agriculture treatment as a categorical factor and the mercury treatment as a continuous factor (using egg THg, dwt, in ppm). Initial models also included lay date as a continuous covariate but it was insignificant and removed from final models. For the field experiment, we used a generalized linear mixed model (GLMM) which included clutch as a random effect and nest layer (top or bottom) as a fixed effect. Because the Hg + Shade treatment group in the laboratory incubation experiment was 100% male, we could not directly test interactive effects of mercury and agriculture and instead, elected to test (1) the effect of agriculture treatment on sex within Ref clutches using a GLMM with agriculture treatment as the single main effect and clutch included as a random effect and (2) the effect of mercury treatment on sex within Open treatment clutches (from Ref and Hg sites) using a general linear model (GLM) with mercury treatment included as a continuous predictor. In our second model clutch was not included as a random effect because it was confounded with clutch mercury concentration, which was a unique value for each clutch.

Using a second set of GLMM models, we examined continuous measures of nest temperature (average temperature, CTE, and thermal variance) and sex ratios in our field incubation experiment. Because there was not enough variation in thermal conditions among clutches in the lab experiment to model thermal parameters as continuous predictors we tested average temperature, CTE, and variance only as categorical predictors in the laboratory experiment.

3. Results

3.1. Nest Microclimate & mercury concentrations

Total egg mercury concentration (THg, in ppm) reported on a dry mass basis (dwt) did not differ between the laboratory and the field experiments (REML: $F_{1,\ 80} = 0.90$, $p = 0.35$). The average egg THg in clutches collected downstream of the mercury source was about 12× higher than clutches collected upstream (Hg = 2.8 ± 0.2 ppm, dwt SE, $n = 23$; Ref = 0.3 ± 0.03 ppm, dwt SE, $n = 20$) but ranged widely within the polluted sites (0.4—5.1 ppm, dwt).

In our Shade plots, vegetative cover increased from < 10% cover and 15 cm height at the start of the study (late May) to over 95% cover, 80% density, and almost 2 m height during and after the average thermosensitive period start date (in early July, Fig. 3). In our Open plots, vegetation never exceeded 25% cover, 20% density, or 20 cm in height (Fig. 3).

The Open and Shade plots initially had the same soil moisture levels but they diverged by June 19 (RM ANOVA: $F_{1,\ 13} = 3.2$, $p < 0.001$, Table 1, Fig. 4A). After June 19, soils in our Open plots consistently contained more moisture than soils in Shade plots (Fig. 4A). Rainfall during the two days before samples was correlated with reduced differences between Shade and Open plots ($R^2 = 0.75$, see inset vertical bars in Fig. 4A). When we converted soil moisture levels to water potentials, the interaction between agriculture treatment and sample date remained significant (RM ANOVA: $F_{1,\ 13} = 2.4$, $p < 0.02$, Table 1, Fig. 4B). Dry conditions in Shade plots on June 19 (2.7% soil moisture) precluded our ability to calculate water potential for that date.

Average temperature and thermal variance (s^2 of temperature) in field nests were affected by the interaction between the agriculture treatment and sample date, both at the top and bottom of nests (RM ANOVA: in both cases $p < 0.01$, Table 1). Average daily temperature became lower in Shade plots than in Open plots on June 19th at the top of nests, and on June 27th at the bottom of nests (Fig. 5A and B). For

Table 1
Repeated Measures ANOVAs examining impacts of the agriculture treatment on the hydric and thermal characteristics of field plots and nests during the 15 sampling dates from May 26 to September 23, 2014 (spaced eight days apart). For within subject effects, *P*-values are from univariate Huynh-Feldt epsilon corrected tests. Significant tests between Open and Shade plots are shown in bold font.

	Agriculture		Sample date		Ag.*Sample date	
Soil moisture	$F_{1,8} = 6.8$	**$P = 0.03$**	$F_{1,13} = 48.5$	**$P < 0.01$**	$F_{1,13} = 3.2$	**$P < 0.01$**
Soil water potential	$F_{1,8} = 2.7$	$P = 0.14$	$F_{1,13} = 39.2$	**$P < 0.01$**	$F_{1,13} = 2.4$	**$P = 0.02$**
Avg. temp. (top)	$F_{1,8} = 121$	**$P < 0.01$**	$F_{1,14} = 135$	**$P < 0.01$**	$F_{1,14} = 21.1$	**$P < 0.01$**
Avg. temp. (bottom)	$F_{1,8} = 128$	**$P < 0.01$**	$F_{1,14} = 119$	**$P < 0.01$**	$F_{1,14} = 20.2$	**$P < 0.01$**
Thermal variance (top)	$F_{1,8} = 95.9$	**$P < 0.01$**	$F_{1,14} = 34.3$	**$P < 0.01$**	$F_{1,14} = 12.5$	**$P < 0.01$**
Thermal variance (bottom)	$F_{1,8} = 126$	**$P < 0.01$**	$F_{1,14} = 39.6$	**$P < 0.01$**	$F_{1,3\ 4} = 10.5$	**$P < 0.01$**

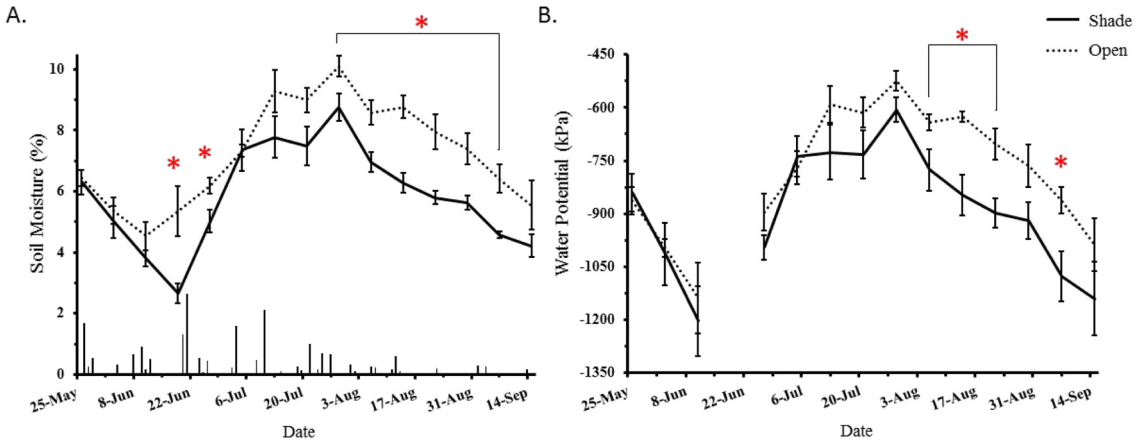

Fig. 4. Average percent soil moisture (A) and water potential (B) in Open (dotted) and Shade (solid) plots throughout incubation. Relative levels of daily precipitation are shown in bars along the x-axis of (A). Water potential estimates are from thermocouple psychrometry. Significant post-hoc tests between Open and Shade plots for each sample date are marked with an asterisks (*), significance was determined at $\alpha = 0.05$. Means ± SE.

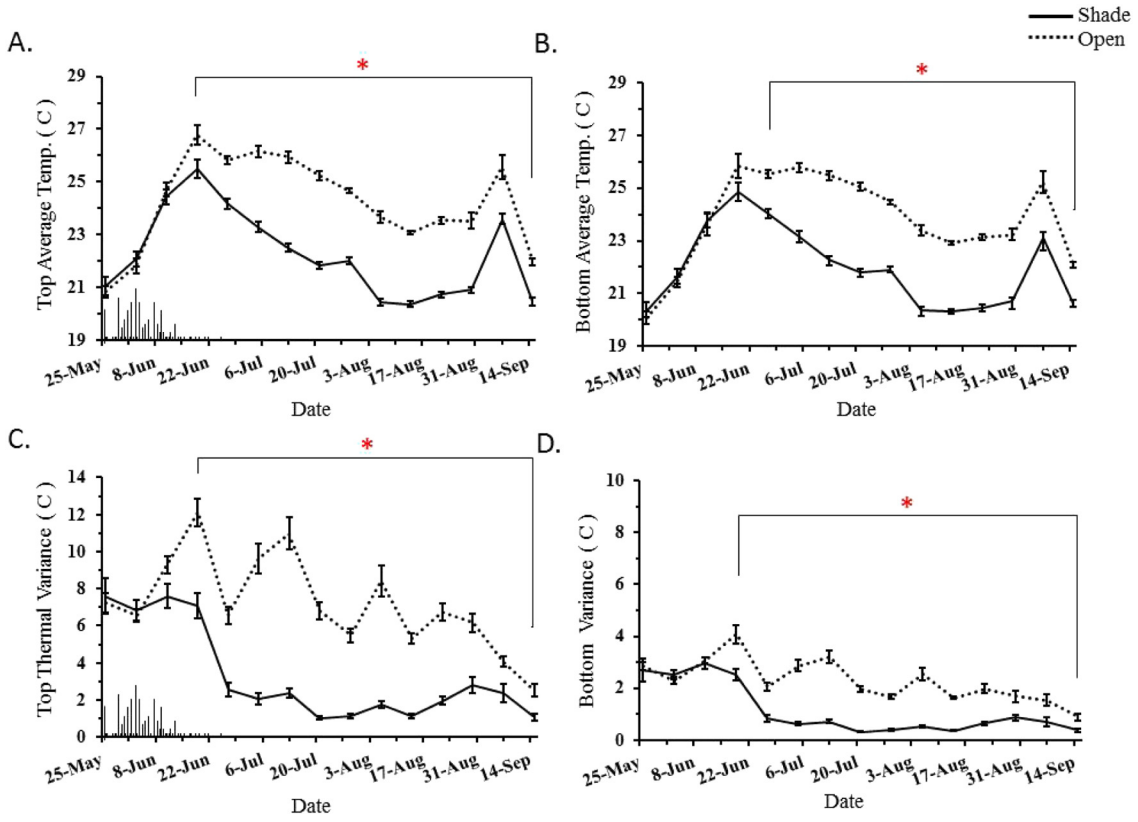

Fig. 5. Average daily temperature at the top (A), bottom (B), and average daily thermal variance at the top (C), and bottom (D) of nests in our field incubation experiment. Black bars on the x-axis show the frequency of oviposition along the South River, VA during 2014. Significant post-hoc tests between Open and Shade plots for each sample date are marked with an asterisks (*), significance was determined at $\alpha = 0.05$. Means ± SE.

both the top and bottom of nests, thermal variance diverged between Shade and Open plots on June 19th (Fig. 5C and D).

During the average thermosensitive period, the thermal variance, average temperature, and CTE of our field nests were lower in the Shade treatment than the Open treatment (LM: in all cases $p < 0.001$, Table 2). Thermal variance at the bottom of nests was lower than at the top (LM: $F_{1,60} = 148$, $p < 0.01$) but nest thermal gradients were larger in the Open than in the Shade treatment. Nest layer interacted with the agriculture treatment to impact CTE: we observed larger differences between the top and bottom of nests in the Open treatment than in the Shade treatment (LM: $F_{1,60} = 9.8$, $p < 0.01$, Table 2).

Actual (laboratory) and target (field) average daily incubation temperatures differed by only 0.30 ± 0.03 $^{\circ}C$ SE and the average hourly standard deviation between actual and target temperatures was 0.86 ± 0.02 °C SE over the course of the laboratory incubation experiment (Tables 2 and 3). During the thermosensitive period in the lab, CTEs were lower than the average of the top and bottom of field nests, by an average of 0.35 °C in Open and 0.05 °C in Shade plots (Fig. 6).

3.2. Offspring sex ratios

In the field experiment, the Shade treatment produced more males

Table 2
Temperature variables observed during the thermosensitive period in agriculture and mercury treatments, in the top and bottom layers of nests in the field incubation experiment. Responses were calculated using daily averages and are shown in $^{\circ}C$. Results of LM analyses with interactions between effects denoted with an asterisk (*), significant results shown in bold, significance was determined at $\alpha = 0.05$. Samples sizes (clutch/layer/treatment groups): Top Layer: Hg/Open: 10, Ref/Open: 8, Hg/Shade: 10, Ref/Shade: 8, Bottom layer: Hg/Open: 9, Ref/Open: 8, Hg/Shade: 9, Ref/Shade: 7.

	Avg. temp.		Thermal variance		CTE	
TRT Group	Top	Bottom	Top	Bottom	Top	Bottom
Hg/open	25.3 ± 0.2	25.0 ± 0.2	8.1 ± 0.8	2.4 ± 0.2	27.1 ± 0.2	25.7 ± 0.3
Ref/open	25.4 ± 0.2	25.1 ± 0.2	9.0 ± 0.7	2.6 ± 0.2	27.3 ± 0.3	26.0 ± 0.3
Hg/shade	21.7 ± 0.3	21.6 ± 0.3	1.5 ± 0.1	0.5 ± 0.04	22.2 ± 0.3	21.8 ± 0.3
Ref/shade	21.7 ± 0.3	21.5 ± 0.3	1.6 ± 0.3	0.7 ± 0.2	22.4 ± 0.3	21.7 ± 0.3
TRT effect						
Mercury	$F_{1,60} = 1.0$, $P = 0.32$		$F_{1,60} = 1.5$, $P = 0.22$		$F_{1,60} = 0.6$, $P = 0.43$	
Agriculture	$F_{1,60} = 951$, $\mathbf{P < 0.01}$		$F_{1,60} = 336$, $\mathbf{P < 0.01}$		$F_{1,60} = 823$, $\mathbf{P < 0.01}$	
Ag.*mercury	$F_{1,60} < 0.0$, $P = 0.86$		$F_{1,60} < 0.0$, $P = 0.99$		$F_{1,60} < 0.0$, $P = 0.89$	
Nest layer	$F_{1,60} = 3.1$, $P = 0.09$		$F_{1,60} = 148$, $\mathbf{P < 0.01}$		$F_{1,60} = 41$, $\mathbf{P < 0.01}$	
Layer*Ag.	$F_{1,60} = 0.8$, $P = 0.39$		$F_{1,60} = 1.8$, $P = 0.18$		$F_{1,60} = 9.8$, $\mathbf{P < 0.01}$	
Layer*mercury	$F_{1,60} = 0.01$, $P = 0.93$		$F_{1,60} = 0.4$, $P = 0.56$		$F_{1,60} = 0.2$, $P = 0.67$	

Table 3
Summary temperature variables observed during the thermosensitive period in the laboratory experiment. All responses are calculated using daily averages and are shown in $^{\circ}C$ ± 1 SE. Average thermosensitive period dates were calculated as the middle third of incubation; thermosensitive period dates were 7/13–8/5 in the Open and 7/17–8/12 in the Shade treatments.

	Hg/open	Ref/open	Hg/shade	Ref/shade
Variance	3.3 ± 0.1	3.4 ± 0.1	0.6 ± 0.02	0.6 ± 0.02
AVG. temp	25.1 ± 0.1	25.2 ± 0.1	21.6 ± 0.2	21.5 ± 0.2
CTE	26.1 ± 0.1	26.2 ± 0.1	22.0 ± 0.2	21.9 ± 0.2

than the Open treatment (GLMM: $z = -3.5$, $p < 0.001$, Fig. 7, Table 4) and this difference was greater in the Hg treatment than in the Ref treatment (interaction GLMM: $z = 1.4$, $p = 0.043$). Nest layer also affected hatchling sex; more males were produced at the bottom of nests than at the top (GLMM: $z = -1.2$, $p = 0.023$, Fig. 7, Table 4). Similarly, lower average daily temperatures, CTEs, and thermal variance decreased male production (in all cases $R^2 \geq 0.34$). Two clutches in the Reference treatment group produced more females than expected based on TSD predictions. When these two clutches were excluded from analysis, R^2 values increased (average increase = 0.20 ± 0.01) and regression analyses showed that the bivariate model with mean temperature and thermal variance explained more variation in offspring sex than the univariate mean temperature and thermal variance models (27 and > 1600 times more likely, respectively). However, the mean temperature and thermal variance model only explained slightly more variation in hatchling sex than the CTE model (1.11 times more likely; AIC weight = 0.4579, Table 5).

In the laboratory experiment we were not able to directly test the interactive effect between agriculture and mercury treatments on offspring sex because the Shade + Hg treatment was 100% male. However, patterns were similar to those observed in the field experiment: within the Ref clutches, the Shade treatment produced more males than the Open treatment (GLMM: $z = -2.8$, $p = 0.006$) and within the Open clutches, the Ref treatment produced more males than the Hg treatments (GLM: $z = -0.2$, $P = 0.011$, Table 4).

4. Discussion

The selection of recently planted agricultural fields for nesting by turtles can have substantial impacts on nest microclimate and offspring sex ratios. We demonstrated that: (1) crop growth near turtle nests can decrease moisture, average temperature, and thermal variance inside of nests; (2) the cooling effect of crops causes male-biased offspring sex ratios in a turtle with TSD; and, (3) high levels of maternally transferred mercury interact with the cooling effect of crops to magnify the effect of agriculture on offspring sex ratio. Differences in water availability can also impact sex ratios of turtles (Wyneken and Lolavar, 2015) but because our results were consistent between the field and laboratory experiments, we provide strong and novel evidence that the primary proximate mechanism driving agriculturally-induced changes in

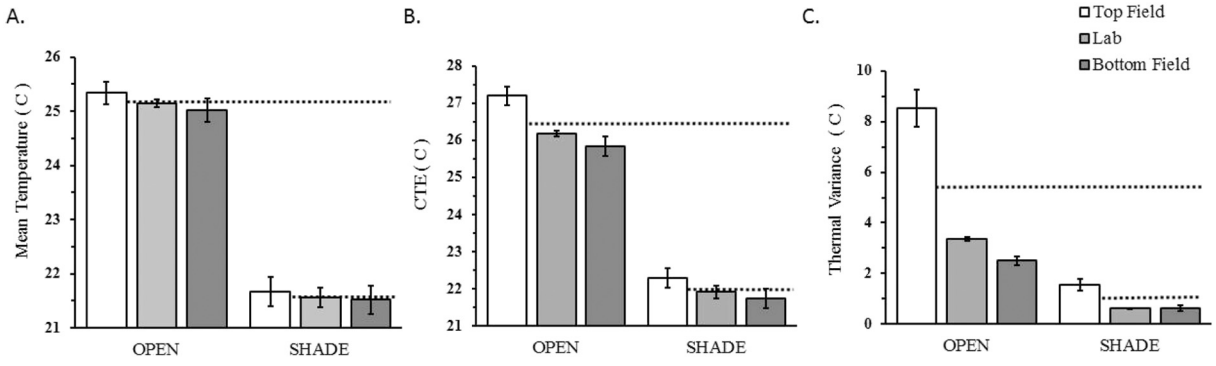

Fig. 6. Comparison of temperature variables in the field and the laboratory incubation experiments during the thermosensitive period. Temperatures from the lab experiment (light grey) are shown between the average values from the top (white) and bottom (dark grey) of nests in the field plots. The average of the top and bottom of field nests (dotted line) shows that (A) mean temperatures in the laboratory experiment were approximately the same as the mean temperatures in the field, but (B) mean CTE in the lab were lower than the average of the top and bottom of field nests, and (C) mean variance was 40% lower than the average of the top and bottom of field nests.

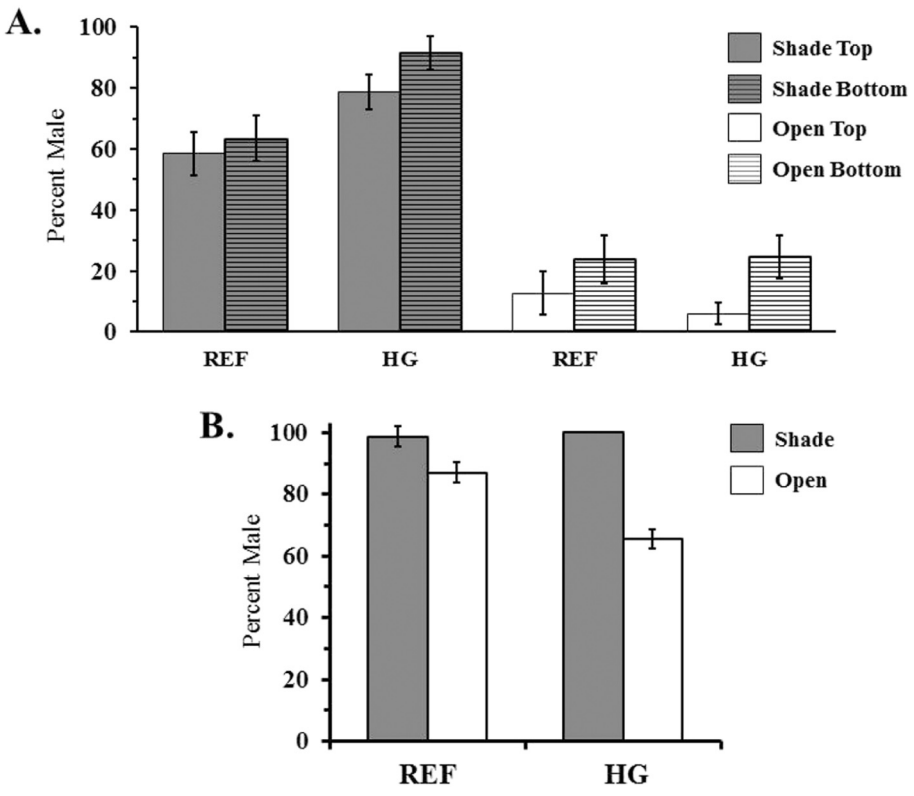

Fig. 7. Relationships between treatments and sex ratios (expressed as percent male [%]) in the field incubation experiment (at the top and bottom of nests; A) and in the laboratory experiment (B). Statistical models treated mercury as a continuous factor but here we show sex ratios as the response and mercury categorically (Ref or Hg) for visual purposes.

Table 4
Summary of treatment effects and offspring sex ratios (percent male) in the field and laboratory experiments (LS Means ± SE). Sex ratios are presented for the top and bottom layers of nests in the field incubation experiment. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by $\alpha = 0.05$. *P*-values show results of logistic regression analyses: results of mercury treatment effects in the laboratory experiment are from GLM analysis; all other results are from GLMM analyses.

	FIELD exp.		LAB exp.
TRT group	Top	Bottom	
Hg/open	6.0 ± 3.6	26.8 ± 7.0	73.1 ± 3.3
Ref/open	12.8 ± 7.2	24.7 ± 7.9	84.0 ± 3.2
Hg/shade	78.7 ± 5.6	91.6 ± 5.4	100 ± 0
Ref/shade	58.5 ± 7.0	63.4 ± 7.4	98.9 ± 3.2
TRT effect			
Mercury	z = −0.02, *P* = 0.919		z = −0.2, ***P* = 0.011**
Agriculture	z = −3.5, ***P* < 0.001**		z = −2.8, ***P* = 0.006**
Ag.*mercury	z = 1.35, ***P* = 0.043**		**
Nest layer	z = −1.2, ***P* = 0.023**		n/a
Layer*Ag.	z = −0.3, *P* = 0.658		n/a
Layer*mercury	z = 0.7, *P* = 0.271		n/a

** The interactive effect of agriculture and mercury treatment on sex could not be tested because all offspring produced were male.

offspring sex ratios is lowered incubation temperature and thermal variance.

Endocrine disrupting chemicals are known to impact offspring sex in reptiles with TSD, yet our study provides the first documentation that mercury can influence sex determination. Reptiles are an understudied group in ecotoxicology (Hopkins, 2000) but the effects of some endocrine disrupting chemicals, mainly dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), on TSD are well studied (Crews et al., 1995; Kohono and Guillette, 2013). In turtles, embryonic exposure to DDT and PCBs can cause abnormal androgen synthesis and lead to sex-reversal to females at male producing temperatures (Bergeron and Crews, 1998; Adams et al., 2016). Mercury is also an endocrine disrupting chemical that affects sex hormone concentrations and impacts the regulation of enzymes involved with steroidogenesis (Tan et al., 2009). The fact that maternally transferred mercury interacted with thermal conditions to influence sex ratios raises important questions about the underlying mechanisms.

We hypothesize two possible mechanisms for the interactive effect between the agriculture and mercury treatments on hatchling sex ratios. First, the thermal effects of the agriculture treatment and the endocrine disrupting effects of mercury may interact to cause a direct change to the pathways of sexual differentiation. In reptiles with TSD, offspring sex is influenced by an interaction between incubation temperature and yolk steroid hormones (Ding et al., 2012; Páez et al., 2015;

Table 5
Results of logistic regression models investigating temperature effects on offspring sex determination in the field incubation experiment. Interactions between effects are denoted with an asterisk (*), significant effects are shown in bold, significance was determined by $\alpha = 0.05$.

Model	Z value	Test stat	AICc	delta-AICc	AIC weight	B	S.E.	Odds ratio
Avg. temp + variance	−4.0, −2.6	$p < 0.01$, $p = 0.011$	374.9	0	0.5060	−0.5543, −0.2585	0.1402, 0.1013	0.57, 0.77
CTE	−8.6	$p < 0.01$	375.1	0.2	0.4579	−0.6933	0.0810	0.51
Avg. temp	−8.5	$p < 0.01$	380.2	5.3	0.0358	−0.8428	0.0989	0.44
Thermal variance	−7.3	$p < 0.01$	389.7	14.8	0.0003	−0.5881	0.0808	0.51

Sun et al., 2016). Consequently, the interaction between the agriculture and mercury treatments in our study may be related to interactions between incubation temperature, yolk hormones (Flood et al., 2013; Warner et al., 2014), and the influence of mercury on the production of estrogens (Zhu et al., 2000) and/or thyroid hormones (Wada et al., 2009, Wada et al., 2010, Meyer et al., 2014; but see Wada et al., 2011). An alternate hypothesis for the interaction between our treatments is the differential mortality of embryos based on their sex (Sakamoto et al., 2001; Bouland et al., 2012; Durant et al., 2016). However, inspection of hatch success and sex ratios among clutches suggest that this explanation is unlikely due to incongruent results between our field and laboratory experiments. Hatching success did not vary significantly between treatments in the field experiment, and was too high (mean: 87%, range: 85–91%) to account for the observed differences in sex ratios (Thompson, 2017). In the laboratory experiment, hatch success in the Shade + Hg treatment group was lower than all other treatment groups (−15% relative to all other groups). Assuming all of the dead embryos were female could have driven the observed interactive effect, but this explanation requires that different mechanisms of sex determination were operating between the lab and field experiment, which seems unlikely. Although the interactive mechanism of mercury and thermal effects on sex ratios is unknown, our results emphasize that major global changes can have complex interactions that affect early vertebrate development in unexpected ways.

Our findings provide a novel illustration of the complexities involved with comparing field and laboratory incubation conditions that can be used to improve future lab-to-field extrapolations. Average temperatures in the laboratory closely matched mean hourly temperatures in field nests, but our laboratory study could not simulate the vertical stratification of temperatures within field nests or the range of thermal profiles among field nests despite being, to our knowledge, the highest resolution incubation regimen used to date. Collapsing variance between top and bottom nest temperatures from the field into nest average temperatures for laboratory simulations proved to have important implications for sex ratios (Figs. 6 and 7). Specifically, temperatures in the laboratory were less likely to cycle above and below the pivotal temperature for sex determination than temperatures in the field. Likewise, some nests in the field were always warmer than the average field nest temperature, and within field nests, some consistently had larger thermal gradients than others despite standardized dimensions and clutch sizes. Because crops substantially lowered thermal variance, the effects of averaging among nests on the Shade laboratory incubation regimen were much less pronounced than effects on the Open laboratory incubation (Fig. 6). Therefore, our work demonstrates the value of coupling laboratory and field studies of TSD, and that extrapolating between these settings can be more appropriate for some contexts (e.g., Shade) than others (e.g., Open) based on the magnitude of thermal fluctuations associated with each treatment.

The use of agricultural fields for nesting is likely maladaptive for turtles. Consistently male-biased offspring sex ratios, like those documented here, could negatively impact population growth rates; turtle population dynamics are more dependent on the number of reproductive females than the number of males (Congdon et al., 1994; Pearse and Avise, 2001; Schwanz et al., 2010). Our study area was planted with corn the year of our study but soy is often planted in this field and has also been linked to male-biased offspring sex ratios in turtles (Freedberg et al., 2011). Agricultural land use can also negatively impact the survival of nesting females, embryos, and hatchlings through a variety of other pathways; agricultural machinery can destroy nests and injure/kill adult and hatchling turtles, dense vegetative root mats can penetrate and destroy eggs, intense canopy cover over nests blocks environmental cues used for hatchling orientation, and nest predation rates are higher relative to non-agricultural nest sites (Saumure and Bider, 1998; Saumure et al., 2007; Pappas et al., 2013; Thompson et al., 2017). Our results suggest that reptile species that are attracted to human-altered environments to nest, particularly when the form of habitat alteration co-occurs with chemical pollutants, should be considered a priority for future research and conservation planning.

Our study provides evidence that conservation planning can be improved by considering how anthropogenic land-use, common environmental pollutants, and climate change interact to influence sex ratios and population dynamics of animals with TSD. We previously demonstrated the occurrence of high density turtle nesting in agricultural fields (Thompson et al., 2017), which is consistent with other studies (Freedberg et al., 2011; Mui et al., 2015). Because shade from agricultural crops causes male-biased sex ratios, particularly in the presence of endocrine disrupting chemicals, the most obvious conservation strategy would be to reduce agricultural use of high density nesting areas. Likewise, increasing the frequency of fallow years (i.e., years when crops are not planted) in high density nesting areas would help mitigate the male-production bias associated with agricultural shade. Alternatively, small-scale removal of crops over nests of turtle species of conservation concern is an additional fast and inexpensive option, similar to what we employed here in cooperation with a local landowner. Taken together, managing high density nesting areas for species of conservation concern through any of these three options will be most valuable if used in combination with predator guards over nests and actions to ensure the nests and hatchlings are not destroyed by agricultural machinery.

Our results also have important conservation implications for understanding the effects of rapid anthropogenic global changes on turtle populations, espeacially in the context of climate change. Although climate change is predicted to increase global temperatures and have a feminizing effect on reptiles with TSD (Böhm et al., 2016), our results add to a growing body of literature showing that nesting turtles are attracted to human disturbed landscapes that have a masculinizing effect on reptiles with TSD (Janzen, 1994; Mrosovsky et al., 1995; Kolbe and Janzen, 2002; Kamel and Mrosovsky, 2006; Freedberg et al., 2011). Thus, our results suggest that the thermal characteristics of habitats that attract nesting turtles, especially human modified habitats, add an additional layer of complexity to current climate projections (Sears and Angilletta, 2015). To make matters worse, male-biased sex ratios in freshwater turtles are commonly attributed to sex-specific road mortality because females are more likely to traverse roads during nesting season (Gibbs and Steen, 2005). Considering that roads are sometimes spatially correlated with both pollution and agricultural land use, conservation planning would benefit by evaluating the interplay between roads and the factors we studied here. Importantly, our results emphasize the need to evaluate the interactions among major global changes on species with TSD to develop effective mitigation measures that will enhance their resilience in the Anthropocene.

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